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<b>(54) Title:</b> HYPERSENSITIVE RESPONSE ELICITOR FROM <i>PSEUDOMONAS SYRINGAE</i> AND ITS USE  <b>(57) Abstract</b>  The present invention is directed to an isolated protein or polypeptide which elicits a hypersensitive response in plants as well as an isolated DNA molecule which encodes the hypersensitive response eliciting protein or polypeptide. This isolated protein or polypeptide and the isolated DNA molecule can be used to impart disease resistance to plants, to enhance plant growth, and/or to control insects on plants. This can be achieved by applying the hypersensitive response elicitor protein or polypeptide in a non-infectious form to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds. Alternatively, transgenic plants or plant seeds transformed with a DNA molecule encoding a hypersensitive response elicitor protein or polypeptide can be provided and the transgenic plants or plants resulting from the transgenic plant seeds are grown under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.		

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## **HYPERSENSITIVE RESPONSE ELICITOR FROM *PSEUDOMONAS SYRINGAE* AND ITS USE**

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5 Science Foundation Grant No. MCB 9631530. The U.S. Government may retain  
certain rights.

This application is entitled to benefit of U.S. Provisional Patent  
Application Serial No. 60/055,107.

### 10 **FIELD OF THE INVENTION**

The present invention relates to a hypersensitive response elicitor from  
*Pseudomonas syringae* and its use.

### 15 **BACKGROUND OF THE INVENTION**

Interactions between bacterial pathogens and their plant hosts generally  
fall into two categories: (1) compatible (pathogen-host), leading to intercellular  
bacterial growth, symptom development, and disease development in the host plant;  
20 and (2) incompatible (pathogen-nonhost), resulting in the hypersensitive response, a  
particular type of incompatible interaction occurring, without progressive disease  
symptoms. During compatible interactions on host plants, bacterial populations  
increase dramatically and progressive symptoms occur. During incompatible  
interactions, bacterial populations do not increase, and progressive symptoms do not  
25 occur.

The hypersensitive response ("HR") is a rapid, localized necrosis that  
is associated with the active defense of plants against many pathogens (Kiraly, Z.,  
"Defenses Triggered by the Invader: Hypersensitivity," pages 201-224 in: Plant  
Disease: An Advanced Treatise, Vol. 5, J.G. Horsfall and E.B. Cowling, ed.  
30 Academic Press New York (1980); Klement, Z., "Hypersensitivity," pages 149-177  
in: Phytopathogenic Prokaryotes, Vol. 2, M.S. Mount and G.H. Lacy, ed. Academic  
Press, New York (1982)). The hypersensitive response elicited by bacteria is readily  
observed as a tissue collapse if high concentrations ( $\geq 10^7$  cells/ml) of a limited  
host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated

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- into the leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum) (Klement, Z., "Rapid Detection of Pathogenicity of Phytopathogenic *Pseudomonads*," Nature 199:299-300; Klement, et al., "Hypersensitive Reaction Induced by Phytopathogenic Bacteria in the Tobacco Leaf," Phytopathology 54:474-477 (1963); Turner, et al., "The Quantitative Relation Between Plant and Bacterial Cells Involved in the Hypersensitive Reaction," Phytopathology 64:885-890 (1974); Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The capacities to elicit the hypersensitive response in a nonhost and be pathogenic in a host appear linked. As noted by Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York, these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the hypersensitive response or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren, P.B., et al., "Gene Cluster of *Pseudomonas syringae* pv. 'phaseolicola' Controls Pathogenicity of Bean Plants and Hypersensitivity on Nonhost Plants," J. Bacteriol. 168:512-22 (1986); Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991)). Consequently, the hypersensitive response may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991); Bonas, U., "*hrp* Genes of Phytopathogenic Bacteria," pages 79-98 in: Current Topics in Microbiology and Immunology: Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms, J.L. Dangel, ed. Springer-Verlag, Berlin (1994)). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem, et al., "Evolutionary Conservation of Pathogenicity Determinants Among Plant and Animal Pathogenic Bacteria," Trends Microbiol. 1:175-180 (1993)). In *E. amylovora*, *P. syringae*, and *P. solanacearum*, *hrp* genes have been shown to control the production and secretion of glycine-rich,

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protein elicitors of the hypersensitive response (He, S.Y., et al. "Pseudomonas Syringae pv. Syringae Harpin<sub>PSS</sub>: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), Wei, Z.-H., et al., "HrpI of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family," J. Bacteriol. 175:7958-7967 (1993); Arlat, M. et al. 5 "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-553 (1994)).

The first of these proteins was discovered in *E. amylovora* Ea32i, a 10 bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei, Z.-M., et al, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992)). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit a hypersensitive response in nonhost tobacco leaves and incite disease symptoms in highly susceptible 15 pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the hypersensitive response in leaves of tobacco, which is not a host of that strain (Arlat, et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-53 (1994)). However, *P.* 20 *solanacearum* *popA* mutants still elicit the hypersensitive response in tobacco and incite disease in tomato. Thus, the role of these glycine-rich hypersensitive response elicitors can vary widely among gram-negative plant pathogens.

Other plant pathogenic hypersensitive response elicitors have been isolated, cloned, and sequenced. These include: *Erwinia chrysanthemi* (Bauer, et. al., 25 "*Erwinia chrysanthemi* Harpin<sub>Ech</sub>: Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995)); *Erwinia carotovora* (Cui, et. al., "The RsmA<sup>+</sup> Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrpN*<sub>Ecc</sub> and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1996)); *Erwinia stewartii* (Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microb. Inter. July 14-19, 1996 and 30 Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on

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Maize," Ann. Mtg. Am. Phytopath. Soc. July 27-31, 1996); and *Pseudomonas syringae* pv. *syringae* (WO 94/26782 to Cornell Research Foundation, Inc.).

The present invention is a further advance in the effort to identify, clone, and sequence hypersensitive response elicitor proteins or polypeptides from  
5 plant pathogens.

### SUMMARY OF THE INVENTION

The present invention is directed to an isolated protein or polypeptide  
10 which elicits a hypersensitive response in plants as well as an isolated DNA molecule which encodes the hypersensitive response eliciting protein or polypeptide.

The hypersensitive response eliciting protein or polypeptide can be used to impart disease resistance to plants, to enhance plant growth, and/or to control insects. This involves applying the hypersensitive response elicitor protein or  
15 polypeptide in a non-infectious form to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

As an alternative to applying the hypersensitive response elicitor protein or polypeptide to plants or plant seeds in order to impart disease resistance, to  
20 enhance plant growth, and/or to control insects on plants, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a hypersensitive response elicitor protein or polypeptide and growing the plant under conditions effective to impart disease resistance, to enhance plant growth, and/or to control  
25 insects in the plants or plants grown from the plant seeds. Alternatively, a transgenic plant seed transformed with the DNA molecule encoding a hypersensitive response elicitor protein or polypeptide can be provided and planted in soil. A plant is then propagated under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

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## BRIEF DESCRIPTION OF THE DRAWINGS.

Figure 1 shows the inhibition of the HR elicited in tobacco by *P. fluorescens*(pHIR11) by expression in trans of the *P. syringae* pv. *tomato* DC3000 *hrpW*. Vector pML 123 derivatives carrying the *hrpW* region, two of which have  $\Omega$  Sp<sup>r</sup> insertions at the indicated site, are shown at the left. The effects on a tobacco leaf of *P. fluorescens* cells carrying these constructs, 24 hr after inoculation at a concentration of  $5 \times 10^8$  cells/ml, are shown at the right.

Figure 2A-C show a physical map of the *P. syringae* pv. *tomato* DC3000 *hrpW* region, its conservation with a corresponding region in *P. syringae* pv. *syringae* B728a, and structural features of HrpW. Figure 2A shows a physical map of the DC3000 genome adjacent to the *hrp* gene cluster with open arrows denoting putative  $\sigma^{54}$  promoters and filled arrows denoting putative HrpL-dependent promoters that control previously defined transcriptional units (Lorang, J.M., et al., Mol. Plant-Microbe Interact., 8:49-57 (1995), which is hereby incorporated by reference). *hrpR* and *hrpS* encode regulatory proteins and are located at the right end of the *hrp* cluster. Numbers in boxes above the map give the percent identity of DC3000 DNA and colinearly arranged partial B728a DNA sequences. In Figure 2B, the diagram of HrpW indicates the hypersensitive response elicitor-like and pectate lyase ("Pel")-like domains. The PCR subclone-generated His<sub>6</sub>-tagged hypersensitive response elicitor domain fragment encompasses amino acids 1-186; the His<sub>6</sub>-tagged Pel domain has amino acids 187-425. Figure 2C shows the sequence of the region in the middle of HrpW that contains 6 glycine-rich repeats (see box), with similar repeats in the HrpZ proteins from *P. syringae* pv. *tomato* ("Pto"), and *P. syringae* pv. *syringae* ("Pss") aligned below. Dashes were introduced where necessary to preserve alignment.

Figure 3 shows the hybridization of *hrpW* under high stringency conditions to total DNA from other bacterial plant pathogens. DNA from the indicated pathogens was isolated, digested with *Eco*RI, resolved on a 0.5% agarose gel, transferred to an Immobilon-N membrane, and hybridized with a <sup>32</sup>P-labeled *hrpW* subclone at 62°C. Abbreviations: Pto, *P. syringae* pv. *tomato*; Psy, *P. syringae* pv. *syringae*; Pgy, *P. syringae* pv. *glycinea*; Ppp, *P. syringae* pv. *papulans*; Ppi, *P. syringae* pv. *pisi*; Pph, *P. syringae* pv. *phaseolicola*; Pta, *P. syringae* pv. *tabaci*; Pvf,

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*P. viridiflava*; Rso, *Ralstonia solanacearum*; Xam, *Xanthomonas campestris* pv. *amoraciae*; Xvs, *X. campestris* pv. *vesicatoria*; Ea, *Erwinia amylovora*; Eca, *E. carotovora*; Ech, *E. chrysanthemi*.

Figures 4A-B show SDS-PAGE and immunoblot analysis of preparations containing HrpW and its hypersensitive response elicitor domain and Pel domain fragments. In Figure 4A, His<sub>6</sub>-tagged full-length HrpW and the two domain fragments were partially purified by Ni-NTA chromatography, separated by SDS-PAGE, and stained with Coomassie Blue R250. The arrow indicates the full-length HrpW, which is produced in very low amounts. Lanes: 1, Pel domain fragment; 2, hypersensitive response elicitor domain fragment; 3, HrpW. In Figure 4B, the same HrpW derivatives were also visualized on immunoblots with anti-HrpW antibodies used in conjunction with the Western Light chemiluminescence assay. Lanes: 4, Pel domain fragment; 5, hypersensitive response elicitor domain fragment; 6, HrpW.

Figure 5 shows the elicitation in tobacco leaves of active tissue death indicative of the HR by cell-free preparations containing HrpW and the N-terminal fragment. The protein preparations analyzed in Fig. 4 were infiltrated into tobacco leaves, in some cases with 1.0 mM Lanthanum chloride. Leaves were photographed 48-hr later. Panels: A., *P. syringae* pv. *syringae* 61 HrpZ (0.12 µg/ml); B, HrpW; C, harpin domain fragment of HrpW (0.22 µg/ml); D, HrpZ + lanthanum chloride; E, HrpW + lanthanum chloride; F, Pel domain fragment of HrpW (1.40 µg/ml).

## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an isolated DNA molecule having a nucleotide sequence of SEQ. ID. No. 1 as follows:

	TCCA	CTTC	GC	TGAT	TTTG	AA	ATTG	GCAG	AT	TCAT	AGAA	AC	GTTC	AGGT	GT	GGAA	ATCAGG	60	
30	CTGA	GTGC	GC	AGAT	TTCG	TT	GATA	AGGG	TG	TGGT	ACTG	GGT	CATT	GTTG	GT	CATT	TC	AAGG	120
	CCTC	TGAG	TG	CGGT	GCGG	AG	CAAT	ACCA	GT	CTTC	CTG	CTG	GCGT	GTGC	AC	ACTG	AGT	CGC	180
	AGGC	ATAG	GC	ATTT	CAGT	TC	CTTG	CGTT	GG	TTGGG	CATAT	AAAA	AAAG	GA	ACTTTT	AAAA			240
35	ACAG	TGCA	AT	GAGAT	GCCG	G	CAAA	ACGG	GA	ACCGG	TCGCT	GCGC	TTTG	CC	ACTC	ACTT	CG		300
	AGCA	AGCT	CA	ACCC	CAAC	A	TCCAC	ATCCC		TATCG	AACG	G	ACAG	CGAT	AC	GGCC	ACTT	GC	360
	TCTG	GTAAC		CCTG	GAGC	TG	GCGT	CGGT	CC	AATTG	CCCC	AC	TTAG	CGAG	GT	AACG	CAGC	AT	420



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GAGCATCGGC ATCACACCCC GGCCGCAACA GACCACCACG CCACTCGATT TTTCGGCGCT 480  
 AAGCGGCAAG AGTCCTCAAC CAAACACGTT CGGCGAGCAG AACACTCAGC AAGCGATCGA 540  
 5 CCCGAGTGCA CTGTTGTTTCG GCAGCGACAC ACAGAAAGAC GTCAACTTCG GCACGCCCGA 600  
 CAGCACCGTC CAGAATCCGC AGGACGCCAG CAAGCCCAAC GACAGCCAGT CCAACATCGC 660  
 10 TAAATTGATC AGTGCATTGA TCATGTCGTT GCTGCAGATG CTCACCAACT CCAATAAAAA 720  
 GCAGGACACC AATCAGGAAC AGCCTGATAG CCAGGCTCCT TTCCAGAACA ACGGCGGGCT 780  
 CGGTACACCG TCGGCCGATA GCGGGGGCGG CGGTACACCG GATGCGACAG GTGGCGGGCGG 840  
 15 CGGTGATACG CCAAGCGCAA CAGGCGGTGG CGGCGGTGAT ACTCCGACCG CAACAGGCGG 900  
 TGGCGGCAGC GGTGGCGGCG GCACACCAC TGCAACAGGT GGCGGCAGCG GTGGCACACC 960  
 20 CACTGCAACA GGCGGTGGCG AGGGTGGCGT AACACCGCAA ATCACTCCGC AGTTGGCCAA 1020  
 CCCTAACCGT ACCTCAGGTA CTGGCTCGGT GTCGGACACC GCAGGTTCTA CCGAGCAAGC 1080  
 CGGCAAGATC AATGTGGTGA AAGACACCAT CAAGGTCGGC GCTGGCGAAG TCTTTGACGG 1140  
 25 CCACGGCGCA ACCTTCACTG CCGACAAATC TATGGGTAAC GGAGACCAGG GCGAAAATCA 1200  
 GAAGCCCATG TTCGAGCTGG CTGAAGGCGC TACGTTGAAG AATGTGAACC TGGGTGAGAA 1260  
 30 CGAGGTCGAT GGCATCCACG TGAAAGCCAA AAACGCTCAG GAAGTCACCA TTGACAACGT 1320  
 GCATGCCCAG AACGTCGGTG AAGACCTGAT TACGGTCAAA GGCGAGGGAG GCGCAGCGGT 1380  
 CACTAATCTG AACATCAAGA ACAGCAGTGC CAAAGGTGCA GACGACAAGG TTGTCCAGCT 1440  
 35 CAACGCCAAC ACTCACTTGA AAATCGACAA CTTCAAGGCC GACGATTTTCG GCACGATGGT 1500  
 TCGCACCAAC GGTGGCAAGC AGTTTGATGA CATGAGCATC GAGCTGAACG GCATCGAAGC 1560  
 40 TAACCACGGC AAGTTCGCCC TGGTGAAGG CGACAGTGAC GATCTGAAGC TGGCAACGGG 1620  
 CAACATCGCC ATGACCGACG TCAAACACGC CTACGATAAA ACCCAGGCAT CGACCCAACA 1680  
 CACCGAGCTT TGAATCCAGA CAAGTAGCTT GAAAAAGGG GGTGGACTC 1729  
 45

This DNA molecule is known as the dspE gene. This isolated DNA molecule of the  
 present invention encodes a protein or polypeptide which elicits a plant pathogen's  
 hypersensitive response having an amino acid sequence of SEQ. ID. No. 2 as follows:

50 Met Ser Ile Gly Ile Thr Pro Arg Pro Gln Gln Thr Thr Thr Pro Leu  
 1 5 10 15  
 55 Asp Phe Ser Ala Leu Ser Gly Lys Ser Pro Gln Pro Asn Thr Phe Gly  
 20 25 30

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Glu Gln Asn Thr Gln Gln Ala Ile Asp Pro Ser Ala Leu Leu Phe Gly  
 35 40 45  
 Ser Asp Thr Gln Lys Asp Val Asn Phe Gly Thr Pro Asp Ser Thr Val  
 5 50 55 60  
 Gln Asn Pro Gln Asp Ala Ser Lys Pro Asn Asp Ser Gln Ser Asn Ile  
 65 70 75 80  
 10 Ala Lys Leu Ile Ser Ala Leu Ile Met Ser Leu Leu Gln Met Leu Thr  
 85 90 95  
 Asn Ser Asn Lys Lys Gln Asp Thr Asn Gln Glu Gln Pro Asp Ser Gln  
 100 105 110  
 15 Ala Pro Phe Gln Asn Asn Gly Gly Leu Gly Thr Pro Ser Ala Asp Ser  
 115 120 125  
 Gly Gly Gly Gly Thr Pro Asp Ala Thr Gly Gly Gly Gly Asp Thr  
 20 130 135 140  
 Pro Ser Ala Thr Gly Gly Gly Gly Gly Asp Thr Pro Thr Ala Thr Gly  
 145 150 155 160  
 25 Gly Gly Gly Ser Gly Gly Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly  
 165 170 175  
 Ser Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly Glu Gly Gly Val Thr  
 180 185 190  
 30 Pro Gln Ile Thr Pro Gln Leu Ala Asn Pro Asn Arg Thr Ser Gly Thr  
 195 200 205  
 Gly Ser Val Ser Asp Thr Ala Gly Ser Thr Glu Gln Ala Gly Lys Ile  
 35 210 215 220  
 Asn Val Val Lys Asp Thr Ile Lys Val Gly Ala Gly Glu Val Phe Asp  
 225 230 235 240  
 40 Gly His Gly Ala Thr Phe Thr Ala Asp Lys Ser Met Gly Asn Gly Asp  
 245 250 255  
 Gln Gly Glu Asn Gln Lys Pro Met Phe Glu Leu Ala Glu Gly Ala Thr  
 260 265 270  
 45 Leu Lys Asn Val Asn Leu Gly Glu Asn Glu Val Asp Gly Ile His Val  
 275 280 285  
 Lys Ala Lys Asn Ala Gln Glu Val Thr Ile Asp Asn Val His Ala Gln  
 50 290 295 300  
 Asn Val Gly Glu Asp Leu Ile Thr Val Lys Gly Glu Gly Gly Ala Ala  
 305 310 315 320

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This protein or polypeptide is about 42.9 kDa.

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portions of the protein. These then would be cloned into an appropriate vector for increased expression of a truncated peptide or protein.

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the elicitor being produced. Alternatively, subjecting a full length elicitor to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydrophobic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

Suitable DNA molecules are those that hybridize to a DNA molecule comprising a nucleotide sequence of SEQ. ID. No. 1 under stringent conditions. An example of suitable high stringency conditions is when hybridization is carried out at 65°C for 20 hours in a medium containing 1M NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% sodium dodecyl sulfate, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 µg/ml *E. coli* DNA. However, any DNA molecules hybridizing to a DNA molecule comprising the nucleotide sequences of SEQ. ID. No. 1, under such stringent conditions must not be identical to the nucleic acids encoding the hypersensitive response elicitor proteins or polypeptides of *E. amylovora* (as disclosed by Wei, Z.-M., et al, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference), *Erwinia chrysanthemi* (as disclosed by Bauer, et. al., "*Erwinia chrysanthemi* Harpin<sub>Ech</sub>: Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995), which is hereby incorporated by reference), *Erwinia carotovora* (as disclosed by Cui, et. al., "The RsmA<sup>-</sup> Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrpN*<sub>Ecc</sub> and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1996), which is hereby incorporated by reference), *Erwinia stewartii* (as disclosed by Ahmad, et. al.,

“Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize,” 8th Int’l. Cong. Molec. Plant-Microb. Inter. July 14-19, 1996 and Ahmad, et. al., “Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize,” Ann. Mtg. Am. Phytopath. Soc. July 27-31, 1996), which are hereby incorporated by reference), and  
5 *Pseudomonas syringae* pv. *syringae* (WO 94/26782 to Cornell Research Foundation, Inc., which is hereby incorporated by reference.

The protein or polypeptide of the present invention is preferably produced in purified form (preferably at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, the protein or polypeptide of the present  
10 invention is secreted into the growth medium of recombinant host cells. Alternatively, the protein or polypeptide of the present invention is produced but not secreted into growth medium. In such cases, to isolate the protein, the host cell (e.g., *E. coli*) carrying a recombinant plasmid is propagated, lysed by sonication, heat, differential pressure, or chemical treatment, and the homogenate is centrifuged to  
15 remove bacterial debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the polypeptide or protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC.

20 The DNA molecule encoding the hypersensitive response elicitor polypeptide or protein can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or  
25 vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form  
30 of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation

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and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccinia virus. Recombinant viruses can be generated by transfection of plasmids into  
5 cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see  
10 "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes." Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced  
15 into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

20 A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems  
25 infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

30 Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from those of procaryotic promotors. Furthermore, eucaryotic promotors and  
5 accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promotors are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes.  
10 Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably  
15 promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote  
20 transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promotors in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promotors may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promotors such as the T7 phage promoter, *lac* promoter,  
25 *trp* promoter, *recA* promoter, ribosomal RNA promoter, the  $P_R$  and  $P_L$  promotors of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lacUV5* (*tac*) promoter or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be  
30 used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operations,

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the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

5                   Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in 10 *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage 15 lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding the hypersensitive response elicitor polypeptide or protein has been cloned into an expression system, it is ready 20 to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The present invention further relates to methods of imparting disease 25 resistance to plants, enhancing plant growth, and/or effecting insect control for plants. These methods involve applying a hypersensitive response elicitor polypeptide or protein in a non-infectious form to all or part of a plant or a plant seed under conditions where the polypeptide or protein contacts all or part of the cells of the plant or plant seed. Alternatively, the hypersensitive response elicitor protein or 30 polypeptide can be applied to plants such that seeds recovered from such plants themselves are able to impart disease resistance in plants, to enhance plant growth, and/or to effect insect control.



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As an alternative to applying a hypersensitive response elicitor polypeptide or protein to plants or plant seeds in order to impart disease resistance in plants, to effect plant growth, and/or to control insects on the plants or plants grown from the seeds, transgenic plants or plant seeds can be utilized. When utilizing

5 transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein and growing the plant under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, and/or to control insects.

Alternatively, a transgenic plant seed transformed with a DNA molecule encoding a

10 hypersensitive response elicitor polypeptide or protein can be provided and planted in soil. A plant is then propagated from the planted seed under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, and/or to control insects.

The embodiment of the present invention where the hypersensitive

15 response elicitor polypeptide or protein is applied to the plant or plant seed can be carried out in a number of ways, including: 1) application of an isolated elicitor polypeptide or protein; 2) application of bacteria which do not cause disease and are transformed with genes encoding a hypersensitive response elicitor polypeptide or protein; and 3) application of bacteria which cause disease in some plant species (but

20 not in those to which they are applied) and naturally contain a gene encoding the hypersensitive response elicitor polypeptide or protein.

In one embodiment of the present invention, the hypersensitive response elicitor polypeptide or protein of the present invention can be isolated from *Pseudomonas syringae* pv. *tomato* as described in the Examples *infra*. Preferably,

25 however, the isolated hypersensitive response elicitor polypeptide or protein of the present invention is produced recombinantly and purified as described *supra*.

In other embodiments of the present invention, the hypersensitive response elicitor polypeptide or protein of the present invention can be applied to plants or plant seeds by applying bacteria containing genes encoding the

30 hypersensitive response elicitor polypeptide or protein. Such bacteria must be capable of secreting or exporting the polypeptide or protein so that the elicitor can contact plant or plant seed cells. In these embodiments, the hypersensitive response elicitor

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polypeptide or protein is produced by the bacteria *in planta* or on seeds or just prior to introduction of the bacteria to the plants or plant seeds.

In one embodiment of the bacterial application mode of the present invention, the bacteria do not cause the disease and have been transformed (e.g.,  
5 recombinantly) with genes encoding a hypersensitive response elicitor polypeptide or protein. For example, *E. coli*, which does not elicit a hypersensitive response in plants, can be transformed with genes encoding a hypersensitive response elicitor polypeptide or protein and then applied to plants. Bacterial species other than *E. coli* can also be used in this embodiment of the present invention.

10 In another embodiment of the bacterial application mode of the present invention, the bacteria do cause disease and naturally contain a gene encoding a hypersensitive response elicitor polypeptide or protein. Examples of such bacteria are noted above. However, in this embodiment, these bacteria are applied to plants or their seeds which are not susceptible to the disease carried by the bacteria. For  
15 example, *Pseudomonas syringae* pv. *tomato* causes disease in tomato but not in beans. However, such bacteria will elicit a hypersensitive response in beans. Accordingly, in accordance with this embodiment of the present invention, *Pseudomonas syringae* pv. *tomato* can be applied to bean plants or seeds to impart disease resistance, enhance growth, or control insects without causing disease in that species.

20 The method of the present invention can be utilized to treat a wide variety of plants or their seeds to impart disease resistance, enhance growth, and/or control insects. Suitable plants include dicots and monocots. More particularly, useful crop plants can include: alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage,  
25 brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Examples of suitable ornamental plants are:  
30 *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention in imparting disease resistance, absolute

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immunity against infection may not be conferred, but the severity of the disease is reduced and symptom development is delayed. Lesion number, lesion size, and extent of sporulation of fungal pathogens are all decreased. This method of imparting disease resistance has the potential for treating previously untreatable diseases, treating diseases systemically which might not be treated separately due to cost, and avoiding the use of infectious agents or environmentally harmful materials.

The method of imparting pathogen resistance to plants in accordance with the present invention is useful in imparting resistance to a wide variety of pathogens including viruses, bacteria, and fungi. Resistance, *inter alia*, to the following viruses can be achieved by the method of the present invention: *Tobacco mosaic virus* and *Tomato mosaic virus*. Resistance, *inter alia*, to the following bacteria can also be imparted to plants in accordance with present invention: *Pseudomonas solanacearum*, *Pseudomonas syringae* pv. *tabaci*, and *Xanthomonas campestris* pv. *pelargonii*. Plants can be made resistant, *inter alia*, to the following fungi by use of the method of the present invention: *Fusarium oxysporum* and *Phytophthora infestans*.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention to enhance plant growth, various forms of plant growth enhancement or promotion can be achieved. This can occur as early as when plant growth begins from seeds or later in the life of a plant. For example, plant growth according to the present invention encompasses greater yield, increased quantity of seeds produced, increased percentage of seeds germinated, increased plant size, greater biomass, more and bigger fruit, earlier fruit coloration, and earlier fruit and plant maturation. As a result, the present invention provides significant economic benefit to growers. For example, early germination and early maturation permit crops to be grown in areas where short growing seasons would otherwise preclude their growth in that locale. Increased percentage of seed germination results in improved crop stands and more efficient seed use. Greater yield, increased size, and enhanced biomass production allow greater revenue generation from a given plot of land.

Another aspect of the present invention is directed to effecting any form of insect control for plants. For example, insect control according to the present invention encompasses preventing insects from contacting plants to which the

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hypersensitive response elicitor has been applied, preventing direct insect damage to plants by feeding injury, causing insects to depart from such plants, killing insects proximate to such plants, interfering with insect larval feeding on such plants, preventing insects from colonizing host plants, preventing colonizing insects from releasing phytotoxins, etc. The present invention also prevents subsequent disease damage to plants resulting from insect infection.

The present invention is effective against a wide variety of insects. European corn borer is a major pest of corn (dent and sweet corn) but also feeds on over 200 plant species including green, wax, and lima beans and edible soybeans, peppers, potato, and tomato plus many weed species. Additional insect larval feeding pests which damage a wide variety of vegetable crops include the following: beet armyworm, cabbage looper, corn ear worm, fall armyworm, diamondback moth, cabbage root maggot, onion maggot, seed corn maggot, pickleworm (melonworm), pepper maggot, and tomato pinworm. Collectively, this group of insect pests represents the most economically important group of pests for vegetable production worldwide.

The method of the present invention involving application of the hypersensitive response elicitor polypeptide or protein can be carried out through a variety of procedures when all or part of the plant is treated, including leaves, stems, roots, propagules (e.g., cuttings) etc. This may (but need not) involve infiltration of the hypersensitive response elicitor polypeptide or protein into the plant. Suitable application methods include high or low pressure spraying, injection, and leaf abrasion proximate to when elicitor application takes place. When treating plant seeds, in accordance with the application embodiment of the present invention, the hypersensitive response elicitor protein or polypeptide can be applied by low or high pressure spraying, coating, immersion, or injection. Other suitable application procedures can be envisioned by those skilled in the art provided they are able to effect contact of the hypersensitive response elicitor polypeptide or protein with cells of the plant or plant seed. Once treated with the hypersensitive response elicitor of the present invention, the seeds can be planted in natural or artificial soil and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants

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may be treated with one or more applications of the hypersensitive response elicitor protein or polypeptide to impart disease resistance to plants, to enhance plant growth, and/or to control insects on the plants.

The hypersensitive response elicitor polypeptide or protein can be  
5 applied to plants or plant seeds in accordance with the present invention alone or in a mixture with other materials. Alternatively, the hypersensitive response elicitor polypeptide or protein can be applied separately to plants with other materials being applied at different times.

A composition suitable for treating plants or plant seeds in accordance  
10 with the application embodiment of the present invention contains a hypersensitive response elicitor polypeptide or protein in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition contains greater than 500 nM hypersensitive response elicitor polypeptide or protein.

Although not required, this composition may contain additional  
15 additives including fertilizer, insecticide, fungicide, nematocide, and mixtures thereof. Suitable fertilizers include  $(\text{NH}_4)_2\text{NO}_3$ . An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents,  
coating agents, and abrading agents. These materials can be used to facilitate the  
20 process of the present invention. In addition, the hypersensitive response elicitor polypeptide or protein can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

In the alternative embodiment of the present invention involving the  
use of transgenic plants and transgenic seeds, a hypersensitive response elicitor  
25 polypeptide or protein need not be applied topically to the plants or seeds. Instead, transgenic plants transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein are produced according to procedures well known in the art.

The vector described above can be microinjected directly into plant  
30 cells by use of micropipettes to transfer mechanically the recombinant DNA. Crossway, Mol. Gen. Genetics, 202:179-85 (1985), which is hereby incorporated by reference. The genetic material may also be transferred into the plant cell using

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polyethylene glycol. Krens, et al., Nature, 296:72-74 (1982), which is hereby incorporated by reference.

Another approach to transforming plant cells with a gene which imparts resistance to pathogens is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference.

The DNA molecule may also be introduced into the plant cells by electroporation. Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the DNA molecule into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *A. rhizogenes* previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a

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suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Agrobacterium is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

After transformation, the transformed plant cells must be regenerated.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If

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these three variables are controlled, then regeneration is usually reproducible and repeatable.

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard  
5 breeding techniques can be used, depending upon the species to be crossed.

Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedure with the presence of the gene encoding the hypersensitive response elicitor resulting in disease resistance, enhanced plant growth, and/or control of insects on the plant. Alternatively,  
10 transgenic seeds are recovered from the transgenic plants. These seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants. The transgenic plants are propagated from the planted transgenic seeds under conditions effective to impart disease resistance to plants, to enhance plant growth, and/or to control insects. While not wishing to be bound by theory, such disease  
15 resistance, growth enhancement, and/or insect control may be RNA mediated or may result from expression of the elicitor polypeptide or protein.

When transgenic plants and plant seeds are used in accordance with the present invention, they additionally can be treated with the same materials as are used to treat the plants and seeds to which a hypersensitive response elicitor polypeptide or  
20 protein is applied. These other materials, including hypersensitive response elicitors, can be applied to the transgenic plants and plant seeds by the above-noted procedures, including high or low pressure spraying, injection, coating, and immersion. Similarly, after plants have been propagated from the transgenic plant seeds, the plants may be treated with one or more applications of the hypersensitive response elicitor to impart  
25 disease resistance, enhance growth, and/or control insects. Such plants may also be treated with conventional plant treatment agents (e.g., insecticides, fertilizers, etc.).

### EXAMPLES

#### 30 Example 1 - Bacterial Strains, Plasmids, and Media.

*E. coli* strains were routinely grown in LM (Hanahan, D. (1985) in DNA Cloning: A Practical Approach, ed. Glover, D.M. (IRL Press, Oxford), pp. 109-



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135, which is hereby incorporated by reference) or Terrific broth (Sambrook, J., Fritsch, E.F., & Maniatis, T. Molecular Cloning, Second Edition. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), pp. 109-135 (1989)), which is hereby incorporated by reference, at 37°C. The *E. coli* strains primarily used for plasmid constructions were DH5 $\alpha$  and DH5aF'IQ (Life Technologies, Grand Island, NY). For standard DNA manipulations, the pBluescript II vectors from Stratagene (La Jolla, CA) were used. *P. syringae* pv. *tomato* DC3000 (Preston, G., Mol. Plant-Microbe Interact., 8:717-32 (1995), which is hereby incorporated by reference) and *P. fluorescens* 55 (Huang, H.C., J. Bacteriol., 170:4748-56 (1988), which is hereby incorporated by reference) were grown in King's B broth (King, E.O., J. Lab. Med., 22:301-07 (1954), which is hereby incorporated by reference) or in *hrp*-derepressing fructose minimal medium (Huynh, T.V., Science, 245:1374-77 (1989), which is hereby incorporated by reference) at 30°C. Antibiotics were used at the following concentrations ( $\mu$ g/ml): ampicillin, 100; gentamicin, 10; kanamycin, 50; rifampicin, 100; spectinomycin, 50; and tetracycline, 20.

### **Example 2 - DNA Manipulations.**

DNA manipulations and PCR reactions were performed according to standard protocols (Sambrook, J., Fritsch, E.F., & Maniatis, T. Molecular Cloning, Second Edition. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) (1989); Innis, M.A., Gelfand, D.H., Sninsky, J.J., & White, T.J. PCR Protocols. (Academic Press, San Diego) (1990), which are hereby incorporated by reference). Oligonucleotide primers for sequencing or PCR were purchased from Life Technologies. PCR reactions used Pfu polymerase (Stratagene). All DNA sequencing was done at the Cornell Biotechnology Center with an Automated DNA Sequencer, model 373A (Applied Biosystems, Foster City, CA). The DNA sequence was analyzed with Genetics Computer Group version 7.3 (Devereaux, J., Gene, 12:387-95 (1984), which is hereby incorporated by reference) and DNASTAR (Madison, WI) software packages. The DNA sequence of *hrpW* has been deposited in GenBank under the accession number AF005221.

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**Example 3 - Plant Assays**

Tobacco (*Nicotiana tabacum* L. "Xanthi") and tomato (*Lycopersicon esculentum* Mill. "Moneymaker") plants were grown and inoculated with bacteria as described (Gopalan, S., Plant Cell, 8:1095-05 (1996), which is hereby incorporated by reference). For virulence assays, bacterial suspensions containing  $10^4$  cells/ml were infiltrated into tomato leaves and monitored daily over a 5-day period for symptom development and bacterial multiplication.

10 **Example 4 - Isolation of DNA Flanking the *hrp* Cluster in *P. Syringae* pv. *tomato* DC3000.**

Total DNA from *P. syringae* pv. *tomato* DC3000 was partially digested with *Sau*3A, ligated into the *Bam*HI site of cosmid vector pCPP47 (Bauer, D.W., Mol. Plant-Microbe Interact., 10:369-79 (1997), which is hereby incorporated by reference), and packaged into phage particles with the Gigapack III Gold Packaging Extract (Stratagene). About 800 bacterial colonies were transferred to Colony/Plaque Screen Hybridization Membranes (DuPont NEN Research Products, Boston, MA) and probed at high stringency with a  $^{32}$ P-labeled *Pst*I fragment containing *hrpR* from *P. Syringae* pv. *syringae* 61, which yielded one hybridizing cosmid, pCPP2357. A 6.5 kb *Eco*RI fragment from pCPP2357 was subcloned into pML123 (Labes, M., Gene, 89:37-46 (1990), which is hereby incorporated by reference), producing pCPP2373. pCPP2374 and pCPP2375 were constructed by partially digesting pCPP2373 with *Mfe*I and inserting an *Eco*RI fragment carrying the  $\Omega$ Sp<sup>r</sup> fragment from pHP45 $\Omega$  into *hrpW* or transcription unit IV (Prentki, P., Gene, 29:303-13 (1984), which is hereby incorporated by reference). A cosmid library was also made in pCPP47 from total DNA from *P. Syringae* pv. *syringae* B728A using the same strategy.

30 **Example 5 - DNA Gel Blots.**

Total DNA (2  $\mu$ g) was digested with *Eco*RI and separated by electrophoresis on 0.5% agarose gels. DNA was transferred to Immobilon-N Membrane (Millipore Co. Bedford, MA) and hybridized at 62°C for 8 h in HYB-9

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Hybridization Solution (GENTRA Systems, Research Triangle Park, NC), with a 1.3 kb PCR-amplified *hrpW* fragment that was labeled with  $^{32}\text{P}$  using the Prime-It II kit (Stratagene). The membranes were washed 4 times in 1.0% SDS and 1X SSC followed by 2 washes in 1.0% SDS and 0.2X SSC. Membranes were exposed to  
5 OMAT X-ray film for 4 to 12 hr.

#### **Example 6 - Preparation of HrpW and Derivatives.**

The complete coding sequence for HrpW was PCR-amplified from  
10 pCPP2368 with the primers 5N-ATGAGGATCCAGCATCGGCATCACACCC-3N (named W1) (SEQ. ID. No. 3) and  
5N-ATGAAAGCTTAAGCTCGGTGTGTTGGGT-3N (named W2) (SEQ. ID. No. 4) which contained *Bam*HI and *Hind*III sites, respectively. DNA encoding the N-terminal 186 amino acids of HrpW was PCR-amplified from pCPP2368 using the W1  
15 primer and the primer 5N-ATGAAAGCTTGCCACCGCCTGTTGCAGT-3N (SEQ. ID. No. 5) which contained a *Hind*III site. DNA encoding the C-terminal 236 amino acids of HrpW was PCR-amplified from pCPP2368 with the primer 5N-ATGAGGATCCGAGGGTGGCGTAACACCG-3N (SEQ. ID. No. 6) which contained a *Bam*HI site and the W2 primer. Amplified products corresponding to  
20 full-length HrpW, the N-terminal and C-terminal portions of HrpW were directionally cloned into the *Bam*HI and *Hind*III sites of pQE30 (Qiagen) resulting in pCPP2377, pCPP2378, and pCPP2379, respectively. Procedures used to isolate His-tagged proteins using Ni-NTA spin columns (Qiagen) were described (Alfano, J.R., Mol. Microbiol., 19:715-28 (1996), which is hereby incorporated by reference). *E. coli*  
25 ABLE K (Stratagene) grown on M9 medium (Sambrook, J., Molecular Cloning, Second Edition. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), which is hereby incorporated by reference), supplemented with glucose (0.2%), casamino acids (0.02%), and thiamine (1  $\mu\text{g/ml}$ ), was used to obtain His<sub>6</sub>-HrpW because of apparent toxicity. SDS-PAGE and immunoblot analyses, using  
30 previously obtained anti-HrpZ and anti-HrpW antibodies (He. S.Y., Cell, 73:1255-66 (1993); Yuan, J., J. Bacteriol., 178:6399-6402 (1996), which are hereby incorporated by reference) and the Western-Light Chemiluminescent Detection System (Tropix,

Bedford, MA) and OMAT X-ray film (Kodak, Rochester, NY) were performed as before (Alfano, J.R., et al., Mol. Microbiol., 19:715-28 (1996), which is hereby incorporated by reference).

5     **Example 7 - Construction of *hrpZ* and *hrpW* Mutations and Marker-Exchange Mutagenesis of *P. syringae* pv. *tomato* DC3000.**

To construct the *P. syringae* pv. *tomato* DC3000 *hrpZ* mutation, a 603 bp *Cla*I fragment internal to *hrpZ* was deleted from pCPP2334, a LITMUS 28 (new  
10     England Biolabs) derivative that contains *hrpA* and *hrpZ*, producing pCPP2336. An *npIII* derivative lacking a transcriptional terminator was PCR-amplified from pCPP2988 (Alfano, J.R., et al., Mol. Microbiol., 19:715-28 (1996), which is hereby incorporated by reference) with primers 5N-  
CCATCGATGGTGGTGGCGATAGCTAGACTTGG-3N (SEQ. ID. No. 7) and 5N-  
15     CCATCGATGGTCTCGTGATGGCAGGTTG-3N (SEQ. ID. No. 8) and cloned into the unique *Cla*I site of pCPP2336 in the correct orientation. A *Bgl*II/*Hind*III fragment from the resulting construct, pCPP2338, carrying the *hrpZ* mutation was exchanged for the *Bgl*II/*Hind*III fragment, carried in pCPP2340, producing pCPP2342. A 5.3 kb *eco*RI fragment from pCPP2342 that carried the *hrpZ* mutation  
20     was cloned into the broad-host range plasmid, pRK415 (Keen, N.T., et al., Gene, 70:191-97 (1988), which is hereby incorporated by reference), producing pCPP2344. An 8.5 *Eco*RI fragment from pCPP2375 which carried *hrpW* interrupted with an  $\Omega$ Sp<sup>r</sup> fragment was subcloned into pRK415 producing pCPP2376. Separately, pCPP2376 and pCPP2344 were electroporated into *P. syringae* pv. *tomato* DC3000. Loss of the  
25     plasmid and retention of the marker was done as previously described (Alfano, J.R., et al., Mol. Microbiol., 19:715-28 (1996), which is hereby incorporated by reference).

30     **Example 8 - *hrpW* Expressed in trans Eliminates the Ability of *P. fluorescens*(pHIR11) to Elicit the HR.**

To identify any hypersensitive response elicitor-like genes in the *P. syringae* pv. *tomato* DC3000 DNA flanking *hrpR*, cosmid pCPP2357, which contains this region in vector pCPP47, was isolated. A series of subclones in pML123 were constructed and screened for two potential hypersensitive response elicitor

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phenotypes: (i) the ability to promote tobacco HR elicitation activity in *P. fluorescens* cells carrying pCPP2274, a  $\Delta hrpZ$  pHIR11 derivative (Gopalan, S., et al., Plant Cell, 8:1095-105 (1996), which is hereby incorporated by reference), and (ii) interference with the HR elicitation activity of *P. fluorescens* cells carrying wild-type pHIR11 (Alfano, J.R., et al., Mol. Microbiol., 19:715-28 (1996), which is hereby incorporated by reference). No subclones had the first phenotype, but one, pCPP2373, had the second. pCPP2373 contains a 6.5-kb *EcoRI* fragment from pCPP2357 that has transcriptional units IV and V (Lorang, J.M. et al., Mol. Plant-Microbe Interact., 8:49-57 (1995), which is hereby incorporated by reference) and eliminated the HR elicitation activity of *P. fluorescens*(pHIR11) (Fig. 1). To determine which transcriptional unit was responsible for the phenotype, an  $\Omega Sp^I$  fragment was inserted into *MfeI* sites in transcriptional units IV and V to construct pCPP2374 and pCPP2375, respectively. Both plasmids were transformed into *P. fluorescens*(pHIR11) cells, which were then infiltrated into tobacco leaves. Only pCPP2375 blocked HR elicitation (Fig. 1), indicating that transcriptional unit V encoded a protein with one of the characteristics of HrpZ.

**Example 9 - The DNA Sequence of *hrpW* Predicts a Protein with Both Hypersensitive Response Elicitor and Pel Domains.**

The complete DNA sequence of transcriptional unit V was determined, revealing a 1,275-bp ORF that was designated by *hrpW*. The gene is preceded by a consensus *hrp* promoter (Lorang, J.M., et al., Mol. Plant-Microbe Interact., 8:49-57 (1995), which is hereby incorporated by reference), and it is followed by a rho-independent terminator (Fig. 2A). The predicted N-terminal sequence of HrpW matches that of EXP-60, one of the five *P. syringae* pv. *tomato* DC3000 Hrp-secreted proteins identified by Yuan and He (Yuan, J., et al., J. Bacteriol., 178:6399-402 (1996), which is hereby incorporated by reference). *hrpW* is flanked by operons transcribed in divergent directions and appears to be in a monocistronic operon. Like hypersensitive response elicitors, the predicted 42.9 kDa HrpW protein is acidic, glycine-rich, lacks cysteine, and is deficient in aromatic amino acids. The predicted protein sequence of HrpW reveals at least two distinct domains (Fig. 2B). A hypersensitive response elicitor-like domain (amino acids 1-186) is rich in glutamine,

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serine, and glycine. The 119-186 region contains six imperfect glycine-rich repeats with many acidic and polar residues, which align with similar repeats in the HrpZ proteins of *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* (Fig. 2C). The T P S/D A T motif in this region is predicted to have  $\beta$ -sheet structure with one side of the  $\beta$ -sheet having all the threonine and serine residues, and the glycine repeats are predicted to be turns by the Garnier-Robson algorithm (Plasterer, T.N., in Methods in Molecular Biology, Vol. 70. ed. Swindell, S.R. (Humana Press, Totawa, NJ), pp. 227-239 (1997), which is hereby incorporated by reference). The alternating  $\beta$ -sheets and turns could form a  $\beta$ -barrel structure. Database searches using BLAST (Altschul, S.F., et al., J. Mol. Biol., 215:403-10 (1990), which is hereby incorporated by reference) revealed no proteins with significant similarity to the hypersensitive response elicitor domain.

In contrast, the C-terminal 236 amino acids of HrpW are similar to several fungal Pels from *Nectria haematococca* mating type IV (*Fusarium solani* f. sp. *pisi*) and to one bacterial Pel from *E. carotovora*. For example, HrpW shows identities of 32.5% with *N. haematococca* PelC (Guo, W., et al., Arch. Biochem. Biophys., 323:352-60 (1995), which is hereby incorporated reference), and 21.2% with Pel-3 from *E. carotovora* (Liu, Y., et al., Appl. Environ. Microbiol., 60:2545-52 (1994), which is hereby incorporated by reference). The amino acid sequence of Pels in this group is dissimilar from the majority of known Pels, and little is known about the active site or tertiary structure of proteins in this group (Henrissat, B., et al., Plant Physiol., 107:963-76 (1995), which is hereby incorporated by reference).

**Example 10 - hrpW Appears Widely Distributed in Plant Pathogenic Bacteria and is in a Region Conserved between Two *P. syringae* Pathovars.**

The distribution of *hrpW* and the conservation of the *hrpW* region were examined by DNA gel blot and DNA sequence analysis. The *hrpW* ORF was amplified by PCR and used as a probe for high stringency gel blot hybridization with *EcoRI*-digested DNA from representative necrogenic Gram-negative plant pathogens (Fig. 3). The *hrpW* probe hybridized to at least one distinct band for each of the *P. syringae* pathovars tested: *glycinea*, *papulans*, *pisi*, *phaseolicola*, *tabaci*, and *syringae* strains B728a and 61 (weakly). Hybridization was also observed with *P.*

*viridiflava* *Ralstonia* (*Pseudomonas*) *solanacearum* (weakly), and *Xanthomonas campestris* pathovars *amoraciae* and *vesicatoria*. No hybridization was observed with DNA from *Erwinia* spp. This region in *P. syringae* pv. *syringae* B728a was further examined by isolating cosmid pCPP2347, which carries DNA hybridizing with both *hrpR* and *hrpW*. Restriction mapping and partial DNA sequence analysis indicated that this region is highly conserved in these two *P. syringae* pathovars and that the *P. syringae* pv. *syringae* B728a HrpW also carries a Pel domain (Fig. 2A).

**Example 11 - HrpW and its Hypersensitive Response Elicitor Domain Elicit an HR-like Necrosis in Tobacco Leaves, but HrpW and the Pel Domain Lack Detectable Pel activity.**

PCR subclones of *hrpW* were constructed in pQE30 to permit production of derivatives of HrpW and the two domain fragments carrying N-terminal His<sub>6</sub>-tags. These fusion proteins were partially purified by Ni-NTA chromatography and analyzed by SDS-PAGE and by immunoblotting with antibodies raised against *P. syringae* pv. *tomato* DC3000 Hrp-secreted proteins (Fig. 4). Anti-HrpW antibodies did bind to the full-length HrpW and to both fragments, but binding to the hypersensitive response elicitor domain fragment was noticeably weaker.

Transformants producing HrpW were highly unstable in their maintenance of the plasmid. Thus, HrpW levels were quite low, and Ni-NTA chromatography yielded a preparation that was only partially enriched in HrpW. Nevertheless, the HrpW preparation elicited a hypersensitive response ("HR")-like necrosis in tobacco leaves, which visibly differed from the necrosis elicited by the *P. syringae* pv. *syringae* 61 HrpZ only in developing ca. 12 hr later (Fig. 5). The elicitor activity was heat-stable and protease sensitive, and vector control preparations produced no response. The partially purified hypersensitive response elicitor domain fragment also elicited a necrosis that was slightly delayed, and this response, like that elicited by HrpZ, could be inhibited by 1.0 mM lanthanum chloride, a calcium channel blocker (Fig. 5).

Thus, the necrosis elicited by the HrpW harpin domain is an active plant response. In contrast, purified *E. chrysanthemi* PelE, obtained from *E. coli* JA-221(pPEL748) (Keen, N.T., et al., *J. Bacteriol.*, 168:595-606 (1986), which is hereby incorporated by reference) elicited a black, macerated necrosis that was not inhibited by 1.0 mM lanthanum chloride, 50  $\mu$ M sodium vanadate, or 100  $\mu$ M cycloheximide. This is

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consistent with the expectation that pectic enzymes kill by lysis of turgid protoplasts through weakened cell walls rather than by elicitation of cell death programs.

Furthermore, the Pel domain fragment elicited no visible response in the infiltrated tobacco tissue. All three proteins were tested for Pel activity by using the sensitive  
 5 A<sub>230</sub> assay for 4,5-unsaturated pectic products (Collmer, A., et al., Meth. Enzymol., 161:329-35 (1988), which is hereby incorporated by reference). No activity was detected despite trying polygalacturonic acid and a 31% methylesterified derivative as substrates, CaCl<sub>2</sub> and MnCl<sub>2</sub> as cofactors, and several pH levels.

10 **Example 12 - The Ability of a *P. syringae* pv. *tomato* DC3000 *hrpZ* *hrpW* Mutant to Elicit the HR is Substantially Reduced.**

Marker-exchange mutagenesis was used to construct *P. syringae* pv. *tomato* mutants CUCPB5094 ( $\Delta hrpZ::nptII$ ), CUCPB5095 (*hrpW*:: $\Omega Sp^r$  (in abd  
 15 /cycOB5985 \* $\Delta hrpZ::nptII$  *hrpW*:: $\Omega Sp^r$ ). The  $\Delta hrpZ::nptII$  mutation is functionally nonpolar, and all mutant constructions were confirmed with DNA gel blots and immunoblots. Tobacco leaves were infiltrated with *P. syringae* pv. *tomato* DC3000 and the three mutant derivatives at two levels of inoculum and then examined 48-hr later for the percentage of infiltrated tissue that was necrotic (Table 1).

20

**Table 1. Reduced frequency of hypersensitive response elicitation in tobacco leaves by *P. syringae* pv. *Tomato* DC30000 *hrpZ* and *hrpW* mutants**

<i>P. syringae</i> pv. <i>tomato</i> strain	Relevant genotype	Inoculum level (cells/ml)	
		1 x 10 <sup>7</sup>	5 x 10 <sup>7</sup>
DC3000	wild type	17/19 <sup>a</sup>	18/18
CUCPB5094	$\epsilon hrpZ \Delta nptII$	12/15	13/13
CUCPB4096	<i>hrpW</i> $\Omega Sp^r$	13/15	13/13
CUCPB5095	$\epsilon hrpZ \Delta nptII$ <i>hrpW</i> $\Omega Sp^r$	7/19	15/18

25

<sup>a</sup>The number of inoculated panels showing more than 50% collapse relative to the total number inoculated 48 hr after inoculation.



Only the *hrpZ hrpW* mutant was significantly reduced in the frequency with which it elicited a robust HR. To determine if this mutant was reduced in virulence, tomato leaves inoculated with the mutant and wild-type DC3000 were monitored for symptom production and bacterial multiplication over a period of 5 days. No difference was observed. To identify an anticipated second hypersensitive response elicitor in *P. syringae*, DNA in the *P. syringae* pv. *tomato* DC3000 *hrp* gene region was screened for genes with hypersensitive response elicitor-like phenotypes. *hrpW* had the expected but paradoxical phenotype of interfering with HR elicitation when expressed in trans and was found to be identical to the previously identified transcriptional unit V and to encode the previously identified Hrp-secreted protein EXP-60 (Lorang, J.M., et al., Mol. Plant-Microbe Interact., 8:49-57 (1995). ; Yuan, J., et al., J. Bacteriol., 178:6399-402 (1996), which are hereby incorporated by reference). Several characteristics of *hrpW* and its product are relevant to outstanding questions regarding the function of hypersensitive response elicitors, the mechanism by which parasite-promoting "Avr" proteins are transferred through the plant cell wall to the interior of plant cells, and the conservation and organization of virulence loci in plant pathogenic bacteria.

HrpW has several general characteristics of hypersensitive response elicitors, including amino-acid composition, heat-stability, unexpectedly low mobility in SDS-PAGE, and the ability of both full-length and truncated proteins to elicit the HR (Alfano, J.R., et al., Plant Cell, 8:1683-98 (1996); He, S.Y., et al., Cell, 73:1255-66 (1993); Wei, Z.M., et al., Science, 257:85-8 (1992); Alfano, J.R., et al., Mol. Microbiol., 19:715-28 (1996), which are hereby incorporated by reference). HrpW also has 6 glycine-rich repeats that are similar to a repeated sequence found in HrpZ and are reminiscent of the repeat-rich structure of HrpZ (Alfano, J.R., et al., Mol. Microbiol., 19:715-28 (1996), which is hereby incorporated by reference). The general lack of cysteine residues in hypersensitive response elicitors is particularly striking in HrpW, because comparison with the homologous fungal and bacterial Pels reveals that all 6 of the conserved cysteine residues in those proteins have been substituted in HrpW. All of these properties raise the possibility that hypersensitive response elicitors, like the *Salmonella typhimurium* FlgM protein, may be in an unfolded state in the absence of its substrate or target (Daughdrill, G.W., et al., Nature

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Struct. Biol., 4:285-91 (1997), which is hereby incorporated by reference). This appears to be important to FlgM because of spatial constraints on the movement of globular proteins through the flagellum. With hypersensitive response elicitors, an unfolded state is more likely important for penetration into the plant cell wall matrix than for translocation through the Hrp pathway, since several Avr proteins thought to travel the pathway into plant cells are relatively large and cysteine-rich.

The ability of isolated *P. syringae* HrpZ and HrpW proteins to elicit the HR when infiltrated into tobacco leaf tissue may not directly reflect biological function, because the Avr proteins now appear to be both essential and sufficient (once delivered to the plant cytoplasm) for elicitation of the bacterial HR (Alfano, J.R., et al., Plant Cell, 8:1683-98 (1996), which is hereby incorporated by reference). Therefore, the hypersensitivity of many plants to hypersensitive response elicitors may be a by-product of the primary activity of these proteins in locally modifying plant cell wall structure in support of parasite-promoting "Avr" protein delivery. Several lines of evidence suggest that the *P. syringae* hypersensitive response elicitors may be such an extracellular component of the Hrp secretion system: (i) *hrpZ* is located within a *hrp* secretion operon that appears conserved among *P. syringae* pathovars (Preston, G., et al., Mol. Plant-Microbe Interact., 8:717-32 (1995), which is hereby incorporated by reference), and *hrpW* (in contrast to typical *avr* genes) appears to be both conserved and linked to the *hrp* cluster; (ii) whereas Avr proteins appear to be secreted out of the bacterial cytoplasm only upon contact with the host (analogous to the contact-dependent type III secretion of the *Yersinia* Yop effector proteins (Cornelis, G.R., et al., Mol. Microbiol., 23:861-67) (1997), which is hereby incorporated by reference), HrpZ and HrpW are secreted when the Hrp system is transcriptionally activated, suggesting they may be components of the translocation apparatus; (iii) the finding that the expression in trans of either *hrpZ* or *hrpW* inhibits the HR elicitation activity of wild-type bacteria is consistent with hypersensitive response elicitors being components of a stoichiometrically-sensitive protein assembly; (iv) HrpZ associates with the walls rather than the membranes of plant cells, and the protein elicits no response from wall-less protoplasts (Hoyos, M.E., et al., Mol. Plant-Microbe Interact., 9:608-16 (1996), which is hereby incorporated by reference); (v) the presence of a Pel domain

in HrpW strongly suggests interaction with the pectic fraction of the cell wall, which is the component controlling porosity of the matrix (Baron-Epel, O., et al., Planta, 175:389-95 (1988), which is hereby incorporated by reference). The growing evidence that large Avr proteins, e.g., AvrBs3 (125 kDa) (Van den Ackerveken, et al.,  
5 Cell, 87:1307-16 (1996), which is hereby incorporated by reference), are delivered to the plant cell cytoplasm suggests that the Hrp system can open a channel through the plant cell wall.

The HrpW protein has no detectable Pel activity. Pel homologs with no detectable activity *in vitro* are also found in the pollen and style tissues of several  
10 plants, and the conservation of catalytic residues in these proteins suggests a cryptic enzymatic function (Henrissat, B., et al., Plant Physiol., 107:963-76 (1995), which is hereby incorporated by reference). The lack of detectable Pel activity in HrpW is not surprising given the biotrophic parasitism of *P. syringae* and the damaging effects of typical Pels on plant tissues. However, the conservation of the Pel domains of the *P.*  
15 *syringae* and *E. amylovora* HrpW proteins suggests that these proteins do have a Pel-related function. In contrast, the extreme variation in the elicitor-active domains argues against an enzymatic basis for the elicitor activity of the proteins.

Mutation of transcription unit V did not reduce either the HR or virulence phenotypes of *P. syringae* pv. *tomato* DC3000 (Lorang, J.M., et al., Mol.  
20 Plant-Microbe Interact., 8:49-57 (1995), which is hereby incorporated by reference), and the *hrpZ hrpW* mutant was significantly reduced only in its HR phenotype (although virulence assay would likely miss a subtle reduction). One interpretation of these observations is that *P. syringae* produces additional hypersensitive response elicitor-like proteins, analogous to the multiple Pel isozymes of *E. chrysanthemi* and  
25 *E. carotovora* (Barras, F., et al., Annu. Rev. Phytopathol., 32:201-34 (1994), which is hereby incorporated by reference). Perhaps as a result of host-parasite coevolution or of cell wall structural complexity, redundancy (or subtle specialization) may be characteristic of virulence systems that interact extensively with the plant cell wall.

The presence of sequences hybridizing with *hrpW* in several other  
30 plant pathogenic bacteria, particularly *P. viridiflava* and *X. campestris* is significant for several reasons. Since *hrpW* does not hybridize with DNA from *E. amylovora* or *E. carotovora*, which are known to produce a similar hypersensitive response elicitor

and Pel, respectively, the hybridization with *P. viridiflava* and *X. campestris* suggests that these bacteria produce a protein that is highly similar to HrpW. This, in turn, implies that *P. viridiflava* has a Hrp system and that *X. campestris* produces a hypersensitive response elicitor. Although the Hrp system of *X. campestris* has been extensively characterized (Bonas, U. in "Current Topics in Microbiology and Immunology," Vol. 192: Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms, ed. Dangel, J.L. (Springer-Verlag, Berlin), pp. 79-98 (1994), which is hereby incorporated by reference), no hypersensitive response elicitor or any other protein has been found to be secreted by the Hrp system in culture. *hrpW* should be useful as a probe to clone from *X. campestris* a gene encoding such a protein.

The pathogenicity island concept predicts that the *hrp* gene cluster is located in a larger region enriched in virulence related genes (Groisman, E.A., et al, Cell, 87:791-94 (1996); Alfano, J.R., et al., Plant Cell, 8:1683-98 (1996), which are hereby incorporated by reference). Some parts of the virulence region would be expected to carry effector-encoding genes that are "switchable" to permit rapid coevolution of the parasite with the host. The *hrmAlavrPphE* locus at the opposite end of the *hrp* cluster from *hrpR* provides such an example. Other, more conserved regions would carry genes related to essential parasitic functions such as the delivery of effector (e.g., "Avr") proteins to the interior of host cells. Comparison of *P. syringae* pathovars *tomato* and *syringae* indicates that *hrpW* is located in such a region. Collectively, these observations suggest a broadly important role for HrpW in bacterial plant pathogenicity. Although both hypersensitive response elicitors and Avr proteins must travel the type III pathway, they differ strikingly in their structural properties, their ability to be secreted in culture, and their effects on host-range determination and other virulence attributes. The discovery of a *P. syringae* hypersensitive response elicitor with a Pel domain provides further evidence that they also differ in their site of action, with many Avr proteins acting inside plant cells and hypersensitive response elicitor acting outside.

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations

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can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Cornell Research Foundation, Inc.

(ii) TITLE OF INVENTION: HYPERSENSITIVE RESPONSE ELICITOR FROM  
PSEUDOMONAS SYRINGAE AND ITS USE

(iii) NUMBER OF SEQUENCES: 8

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(F) ZIP: 14603

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: US 60/055,107  
(B) FILING DATE: 06-AUG-1997

## (viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: 19603/1742

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1729 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- 37 -

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCTCTGAGTG CGGTGCGGAG CAATACCACT CTTCTGCTG GCGTGTGCAC ACTGAGTCGC	180
AGGCATAGGC ATTTCACTTC CTTGCGTTGG TTGGGCATAT AAAAAAAGGA ACTTTTAAAA	240
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AGCAAGCTCA ACCCAAACA TCCACATCCC TATCGAACGG ACAGCGATAC GGCCACTTGC	360
TCTGGTAAAC CCTGGAGCTG GCGTCGGTCC AATTGCCAC TTAGCGAGGT AACGCAGCAT	420
GAGCATCGGC ATCACACCCC GGCCGCAACA GACCACCAG CCACTCGATT TTTCGGCGCT	480
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CAGCACCGTC CAGAATCCGC AGGACGCCAG CAAGCCCAAC GACAGCCAGT CCAACATCGC	660
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GCAGGACACC AATCAGGAAC AGCCTGATAG CCAGGCTCCT TTCCAGAACA ACGGCGGGCT	780
CGGTACACCG TCGGCCGATA GCGGGGGCGG CGGTACACCG GATGCGACAG GTGGCGGCGG	840
CGGTGATACG CCAAGCGCAA CAGGCGGTGG CGGCGGTGAT ACTCCGACCG CAACAGGCGG	900
TGGCGGCAGC GGTGGCGGCG GCACACCCAC TGCAACAGGT GGCGGCAGCG GTGGCACACC	960
CACTGCAACA GGCAGTGGCG AGGGTGGCGT AACACCGCAA ATCACTCCGC AGTTGGCCAA	1020
CCCTAACCGT ACCTCAGGTA CTGGCTCGGT GTCGGACACC GCAGTTCTA CCGAGCAAGC	1080
CGGCAAGATC AATGTGGTGA AAGACACCAT CAAGGTCGGC GCTGGCGAAG TCTTTGACGG	1140
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GAAGCCCATG TTCGAGCTGG CTGAAGGCGC TACGTTGAAG AATGTGAACC TGGGTGAGAA	1260
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CAACGCCAAC ACTCACTTGA AAATCGACAA CTTCAAGGCC GACGATTTTC GCACGATGGT	1500
TCGCACCAAC GGTGGCAAGC AGTTTGATGA CATGAGCATC GAGCTGAACG GCATCGAAGC	1560
TAACCACGGC AAGTTCGCCC TGGTGAAAAG CGACAGTGAC GATCTGAAGC TGGCAACGGG	1620
CAACATCGCC ATGACCGACG TCAAACACGC CTACGATAAA ACCCAGGCAT CGACCCAACA	1680

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CACCGAGCTT TGAATCCAGA CAAGTAGCTT GAAAAAAGGG GGTGGACTC

1729

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 424 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Glu Gln Asn Thr Gln Gln Ala Ile Asp Pro Ser Ala Leu Leu Phe Gly
          35           40           45

Ser Asp Thr Gln Lys Asp Val Asn Phe Gly Thr Pro Asp Ser Thr Val
          50           55           60

Gln Asn Pro Gln Asp Ala Ser Lys Pro Asn Asp Ser Gln Ser Asn Ile
65           70           75           80

Ala Lys Leu Ile Ser Ala Leu Ile Met Ser Leu Leu Gln Met Leu Thr
          85           90           95

Asn Ser Asn Lys Lys Gln Asp Thr Asn Gln Glu Gln Pro Asp Ser Gln
          100          105          110

Ala Pro Phe Gln Asn Asn Gly Gly Leu Gly Thr Pro Ser Ala Asp Ser
          115          120          125

Gly Gly Gly Gly Thr Pro Asp Ala Thr Gly Gly Gly Gly Gly Asp Thr
          130          135          140

Pro Ser Ala Thr Gly Gly Gly Gly Gly Asp Thr Pro Thr Ala Thr Gly
          145          150          155          160

Gly Gly Gly Ser Gly Gly Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly
          165          170          175

Ser Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly Glu Gly Gly Val Thr
          180          185          190

Pro Gln Ile Thr Pro Gln Leu Ala Asn Pro Asn Arg Thr Ser Gly Thr
          195          200          205

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Gly Ser Val Ser Asp Thr Ala Gly Ser Thr Glu Gln Ala Gly Lys Ile  
 210 215 220  
 Asn Val Val Lys Asp Thr Ile Lys Val Gly Ala Gly Glu Val Phe Asp  
 225 230 235 240  
 Gly His Gly Ala Thr Phe Thr Ala Asp Lys Ser Met Gly Asn Gly Asp  
 245 250 255  
 Gln Gly Glu Asn Gln Lys Pro Met Phe Glu Leu Ala Glu Gly Ala Thr  
 260 265 270  
 Leu Lys Asn Val Asn Leu Gly Glu Asn Glu Val Asp Gly Ile His Val  
 275 280 285  
 Lys Ala Lys Asn Ala Gln Glu Val Thr Ile Asp Asn Val His Ala Gln  
 290 295 300  
 Asn Val Gly Glu Asp Leu Ile Thr Val Lys Gly Glu Gly Gly Ala Ala  
 305 310 315 320  
 Val Thr Asn Leu Asn Ile Lys Asn Ser Ser Ala Lys Gly Ala Asp Asp  
 325 330 335  
 Lys Val Val Gln Leu Asn Ala Asn Thr His Leu Lys Ile Asp Asn Phe  
 340 345 350  
 Lys Ala Asp Asp Phe Gly Thr Met Val Arg Thr Asn Gly Gly Lys Gln  
 355 360 365  
 Phe Asp Asp Met Ser Ile Glu Leu Asn Gly Ile Glu Ala Asn His Gly  
 370 375 380  
 Lys Phe Ala Leu Val Lys Ser Asp Ser Asp Asp Leu Lys Leu Ala Thr  
 385 390 395 400  
 Gly Asn Ile Ala Met Thr Asp Val Lys His Ala Tyr Asp Lys Thr Gln  
 405 410 415  
 Ala Ser Thr Gln His Thr Glu Leu  
 420

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

- 40 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGAGGATCC AGCATCGGCA TCACACCC

28

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATGAAAGCTT AAGCTCGGTG TGTGGGT

28

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGAAAGCTT GCCACCGCCT GTTGCACT

28

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGAGGATCC GAGGGTGGCG TAACACCG

28

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## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCATCGATGG TGGTGGCGAT AGCTAGACTT GG

32

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCATCGATGG TCTCGTGATG GCAGGTTG

28

**WHAT IS CLAIMED:**

1. An isolated DNA molecule encoding a hypersensitive response eliciting protein or polypeptide, wherein the isolated DNA molecule is selected from the group consisting of (a) a DNA molecule comprising a nucleotide sequence of SEQ. ID. No. 1, (b) a DNA molecule encoding a protein comprising an amino acid of SEQ. ID. No. 2, (c) a DNA molecule which hybridizes to a DNA molecule comprising a nucleotide sequence of SEQ. ID. No. 1 under stringent conditions, and (d) a DNA molecule complementary to DNA molecules (a), (b), and (c).
2. An isolated DNA molecule according to claim 1, wherein said DNA molecule is a DNA molecule comprising a nucleotide sequence of SEQ. ID. No. 1.
3. An isolated DNA molecule according to claim 1, wherein said DNA molecule is a DNA molecule encoding protein comprising an amino acid of SEQ. ID. No. 2.
4. An isolated DNA molecule according to claim 1, wherein said DNA molecule is a DNA molecule which hybridizes to a DNA molecule comprising a nucleotide sequence of SEQ. ID. No. 1 under stringent conditions.
5. An isolated DNA molecule according to claim 1, wherein said DNA molecule is a DNA molecule complementary to DNA molecules (a), (b), and (c).
6. An expression vector transformed with the DNA molecule of claim 1.
7. An expression vector according to claim 6, wherein the DNA molecule is in proper sense orientation and correct reading frame.
8. A host cell transformed with the DNA molecule of claim 1.

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9. A host cell according to claim 8, wherein the host cell is selected from the group consisting of a plant cell or a bacterial cell.

10. A host cell according to claim 8, wherein the DNA molecule is transformed with an expression vector.

11. A transgenic plant transformed with the DNA molecule of claim 1.

12. A transgenic plant according to claim 11, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

13. A transgenic plant according to claim 11, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

14. A transgenic plant seed transformed with the DNA molecule of claim 1.

15. A transgenic plant seed according to claim 14, wherein the plant seed is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

16. A transgenic plant seed according to claim 14, wherein the plant seed is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

17. An isolated hypersensitive response eliciting protein or polypeptide selected from the group consisting of a protein or polypeptide having an amino acid comprising SEQ. ID. No. 2, and an amino acid encoded by a nucleic acid which hybridizes to a DNA molecule comprising a nucleotide sequence of SEQ. ID. No. 1.

18. An isolated protein or polypeptide according to claim 17, wherein the protein or polypeptide has an amino acid comprising SEQ. ID. No. 2.

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19. An isolated protein or polypeptide according to claim 17, wherein the protein or polypeptide is encoded by a nucleic acid which hybridizes to a DNA molecule comprising a nucleotide sequence of SEQ. ID. No. 1.

15

20. A method of imparting disease resistance to plants comprising: applying a protein or polypeptide according claim 17 in a non-infectious form to a plant or plant seed under conditions effective to impart disease resistance.

20

21. A method according to claim 20, wherein plants are treated during said applying.

22. A method according to claim 20, wherein plant seeds are treated during said applying, said method further comprising:

25

planting the seeds treated with the hypersensitive response elicitor in natural or artificial soil and

propagating plants from the seeds planted in the soil.

23. A method of enhancing plant growth comprising: applying a protein or polypeptide according claim 17 in a non-infectious form to a plant or plant seed under conditions effective to enhance plant growth.

30

24. A method according to claim 23, wherein plants are treated during said applying.

35

25. A method according to claim 23, wherein plant seeds are treated during said applying, said method further comprising:  
planting the seeds treated with the hypersensitive response  
5 elicitor in natural or artificial soil and  
propagating plants from the seeds planted in the soil.
26. A method of insect control for plants comprising:  
applying a protein or polypeptide according claim 17 in a non-  
10 infectious form to a plant or plant seed under conditions effective to control insects.
27. A method according to claim 26, wherein plants are treated during said applying.
- 15 28. A method according to claim 26, wherein plant seeds are treated during said applying, said method further comprising:  
planting the seeds treated with the hypersensitive response  
elicitor in natural or artificial soil and  
propagating plants from the seeds planted in the soil.
- 20 29. A method of imparting disease resistance to plants comprising:  
providing a transgenic plant or plant seed transformed with a  
DNA molecule according to claim 1 and  
growing the transgenic plant or transgenic plants produced  
25 from the transgenic plant seeds under conditions effective to impart disease resistance.
30. A method according to claim 29, wherein a transgenic plant is provided.
- 30 31. A method according to claim 29, wherein a transgenic plant seed is provided.
32. A method of enhancing plant growth comprising:  
providing a transgenic plant or plant seed transformed with a  
35 DNA molecule according to claim 1 and

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growing the transgenic plant or transgenic plants produced from the transgenic plant seeds under conditions effective to enhance plant growth.

5           33.    A method according to claim 32, wherein a transgenic plant is provided.

          34.    A method according to claim 32, wherein a transgenic plant seed is provided.

10           35.    A method of insect control for plants comprising:  
                  providing a transgenic plant or plant seed transformed with a DNA molecule according to claim 1 and  
                  growing the transgenic plant or transgenic plants produced from the transgenic plant seeds under conditions effective to control insects.

15           36.    A method according to claim 35, wherein a transgenic plant is provided.

20           37.    A method according to claim 35, wherein a transgenic plant seed is provided.

          38.    A composition comprising:  
                  a protein or polypeptide according to claim 17 and  
                  a carrier.

25           39.    A composition according to claim 38 further comprising an additive selected from the group consisting of fertilizer, insecticide, fungicide, nematocide, and mixtures thereof.



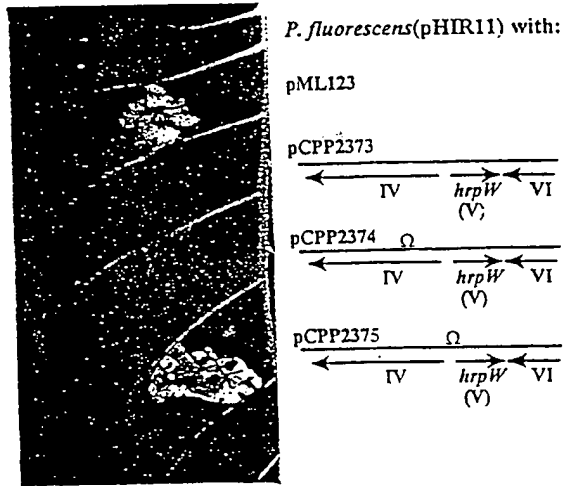
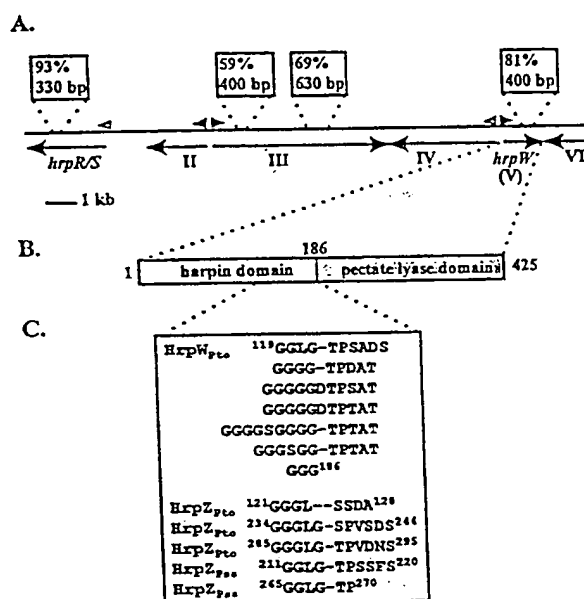


FIGURE 1

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FIGURES 2A, 2B, and 2C

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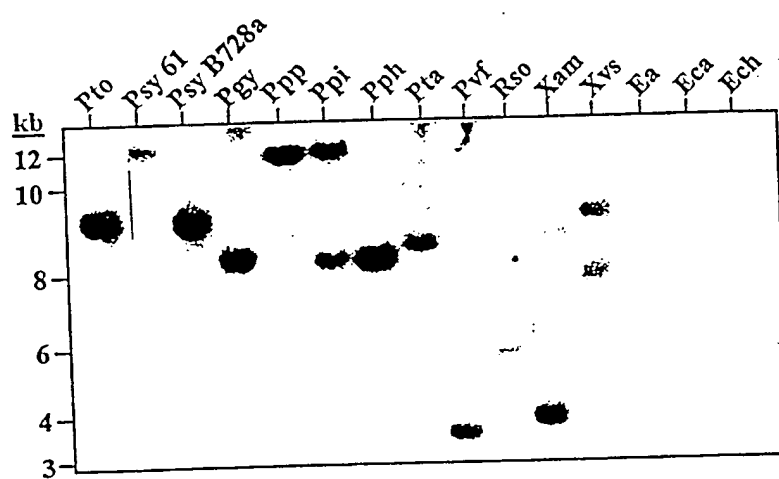
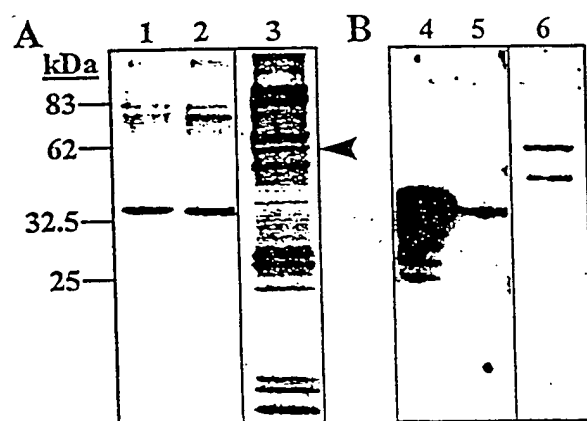


FIGURE 3

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FIGURES 4A and 4B

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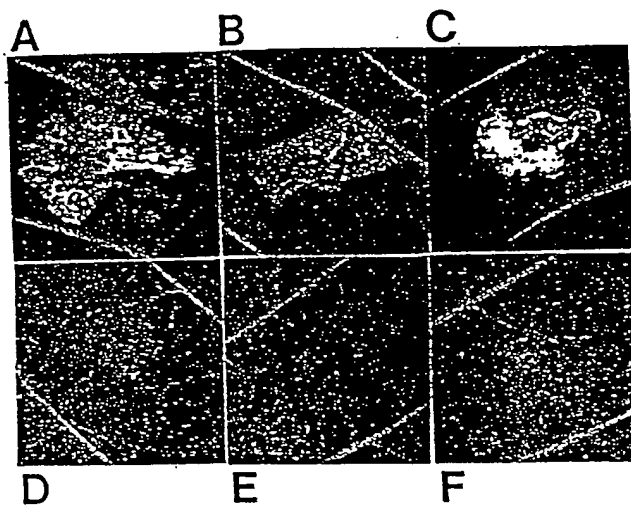


FIGURE 5

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/15501

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A01G 13/00; A61K 35/66; C12N 1/20, C12R 1/18

US CL : 530/350; 536/23.7; 435/874; 800/301

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.7; 435/874; 800/301

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, AGRICOLA, CAPLUS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HE et al. Pseudomonas Syringae Pv. Syringae Harpin: A Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants. Cell. 1993, Vol, 1255, pages 1255-1266, see entire documentation.	1-16, 29-37
Y	BURR et al. Increased Potato Yields by Treatment of Seedpiece with Specific Strains of Pseudomonas Fluorescens and P. Putida. Phytopathology. 1978, Vol. 68, pages 1377-1383, see entire document.	1-16, 29-37



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

24 OCTOBER 1998

Date of mailing of the international search report

19 NOV 1998

Name and mailing address of the ISA/US  
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/15501

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-16,29-37

Remark on Protest

☐  
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/15501

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

**Group I.** Claims 1-16, 29-37 are drawn to a DNA molecule s encoding the hypersensitive eliciting protein, transformation and resulting transgenic plants.

**Group II:** Claims 17-28, 38-39 are drawn to an isolated hypersensitive eliciting response eliciting protein.

The inventions listed as groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The specific technical feature of group 1 is a transgenic plant with improved disease resistance as a result of the expression of transgenes. Second product does not require the special technical features of group 1 because it entails the use of protein and it does not require the particular DNA molecules of group 1. Since group 1 involves the use of expression of transgene in transgenic plants and as such do not share the same technical features. Thus, the claims are not so linked by a special technical feature within the meaning of the PCT Rule 13.2 so as to form a single inventive concept, accordingly, the unity of invention is lacking among all groups.

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